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Genetic variation within and among 22 accessions of three tall larkspur species (*Delphinium* spp.) based on RAPD markers☆

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Abstract

Random amplified polymorphic DNA (RAPD) markers were used to assess genetic diversity in three species of toxic larkspurs (*Delphinium* spp). A total of 184 plants from 22 accessions in five western states were analyzed by 23 RAPD primers that amplified 188 reproducible bands. There were 144 polymorphic bands; 10 shared by *Delphinium glaucum* and *Delphinium occidentale*, eight shared by *Delphinium barbeyi* and *D. glaucum*, and 18 shared by *D. occidentale* and *D. barbeyi*. Thirteen bands were specific for *D. occidentale*, 18 for *D. glaucum* and 19 for *D. barbeyi*. There were 58 bands that were specific for individual accessions and 44 bands that were common to all three species. Some of the species-specific bands were cloned and tested in Southern hybridization. Based on the presence or absence of the 144 polymorhpic RAPD bands in individuals, a dendrogram was generated to assess the genetic similarity among the samples. The cophenetic values were 0.64 between *D. occidentale* and *D. barbeyi*, and 0.55 between the cluster of these two species and *D. glaucum*. These relationships are congruent with those based on morphological characters and support the contention that these are separate species. Understanding the genetic relationships among these three tall larkspur species will provide basic knowledge useful in developing strategies to reduce livestock losses by these poisonous plants.

Keywords: Genetic variation; Random amplified polymorphic DNA; Species-specific; Larkspur; Poisonous plants

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1. Introduction

Larkspur (*Delphinium* spp.) plants containing toxic norditerpenoid alkaloids (Pfister et al., 1993) pose a serious problem on rangelands in the western US. Annual fatalities due to larkspur poisoning average about 5% of cattle grazing larkspur-infested areas (Pfister et al., 1997). Larkspurs are grouped into tall, plains and low larkspurs according to their growth characteristics (Knight and Pfister, 1997). Tall larkspurs include *Delphinium barbeyi* (L) Huth, *Delphinium glaucescens* Rydb., *Delphinium glaucum* Wats., and *Delphinium occidentale* S. Watts, that generally grow at higher elevation (>2000 m) and reach 90–200 cm in height. Three of the tall larkspurs, *D. barbeyi*, *D. glaucum*, and *D. occidentale*, have similar leaf structure and growth form, but are distinguished by the type and amount of pubescence on the inflorescence and by the shape and arrangement of the flowers (Ewan, 1945; Table 1 in part 1 of this series and issue). There seems to be some taxonomic uncertainty regarding the distinction of these larkspur species. Warnock (1995) regrouped plants that were historically classified as *D. occidentale* into *D. glaucum*, and restricted the classification of *D. barbeyi* to only those plants from a small region across the top of the Wasatch plateau in Utah. *D. occidentale* was defined as those plants being hybrids of *D. barbeyi* and *D. glaucum*.

Table 1
Accessions of three species of larkspur collected from different locations in the western US

No.	Species	Code	Location	Date of collection	No. of samples
1	*D. barbeyi*	B1	Cedar City, UT	7/16/98	10
2	*D. barbeyi*	B2	Salina, UT	7/23/98	10
3	*D. barbeyi*	B3	Manti, UT	8/13/98	10
4	*D. barbeyi*	B4	Montrose, CO	7/25/98	8
5	*D. barbeyi*	B5	Crested Butte, CO	7/23/98	12
6	*D. barbeyi*	B6	Yampa, CO	7/29/98	10
7	*D. barbeyi*	B7	Manti, UT	1997	8
8	*D. o–b* hybrid	H1	Yampa, CO	7/29/98	10
9	*D. o–b* hybrid	H2	Salina, UT	7/23/98	10
10	*D. o–b* hybrid	H3	Fairview, UT	8/18/98	10
11	*D. occidentale*	O1	Logan, UT	8/7/98	10
12	*D. occidentale*	O2	Mendon, UT	8/4/98	5
13	*D. occidentale*	O3	Clifton, ID	8/4/98	5
14	*D. occidentale*	O4	Park valley, UT	8/11/98	10
15	*D. occidentale*	O5	Oakley, ID	7/22/98	10
16	*D. occidentale*	O6	Jackson, WY	8/5/98	10
17	*D. occidentale*	O7	Alpine, WY	8/5/98	10
18	*D. occidentale*	O8	Franklin, ID	8/7/98	3
19	*D. occidentale*	O9	Oakley, ID	1997	2
20	*D. glaucum*	G1	Sonora, CA	8/12/98	3
21	*D. glaucum*	G2	Placerville, CA	8/12/98	10
22	*D. glaucum*	G3	Carson Pass, CA	1997	8
Total		22	20		184

There is evidence that tall larkspur plants differ in toxicity (Ralphs et al., 1997), and based on their historical classification, selective management recommendations have been made to prevent livestock poisonings. There is a clear need to determine if these entities are genetically distinct and whether their subsequent toxicity is sufficiently distinct to warrant different management recommendations.

D. barbeyi, *D. glaucum*, and *D. occidentale* have the same number of chromosomes ($2n$=16) and similar chromosome size (K.B. Jensen, personal communication). Thus, species-specific molecular markers will be useful in the identification of larkspur species and their hybrids. Random amplified polymorphic DNA (RAPD; Welsh and McClelland, 1990; Williams et al., 1990) has been used for analyzing genetic diversity within and/or among plant species, cultivars, genomes, chromosomes and genes (Quiros et al., 1991; Hu and Quiros, 1991; Mulcahy et al., 1992; Schachermayr et al., 1994; Wei and Wang, 1995; Svitachev et al., 1998). We report here on the molecular genetic relationship of three of the tall larkspur species and the identification of species-specific DNA markers.

2. Materials and methods

2.1. Plant materials

One hundred and eighty-four individual plants of *D. occidentale*, *D. glaucum*, and *D. barbeyi*, a total of 22 accessions, collected in 1997 and 1998 from the Rocky Mountain regions of Utah, Idaho, Wyoming, California, and Colorado were used in this study (Table 1, Fig. 1). Plant samples were collected at nine locations in Utah, Idaho and Wyoming for *D. occidentale*, at three locations in California for *D. glaucum*, and at seven locations in Utah and Colorado for *D. barbeyi*. Additionally, one accession from Yampa, CO, and two from Utah (Salina and Fairview) was identified as putative interspecific hybrids between *D. occidentale* and *D. barbeyi*.

2.2. Samples collection and template DNA preparation

The young leaves of individual plants were collected, placed in plastic bags and immediately frozen on dry ice for transportation to the laboratory. After 1–2 days, samples were freeze-dried and stored in plastic bags at ambient temperature (~22°C).

The genomic DNA of individual plants was extracted from 100 mg dry leaves using the modified CTAB protocols, quantified by a TKO100 DNA fluorometer and diluted to a final concentration of 5 ng/μl. Genomic DNA of *D. occidentale* is rich in polysaccharides that interfere with the PCR reactions. Removal of polysaccharides was accomplished by using the OmniPrep kit (GenoTech, St. Louis, MO) for DNA extraction. Equal amounts of each individual's DNA from the three species collected in 1997 were mixed to make the bulked DNA for screening and selecting random primers (Operon Technologies) that produced polymorphic fragments among or between species. All the 184 individuals' genomic DNA samples collected in two years were used to select species-specific DNA markers.

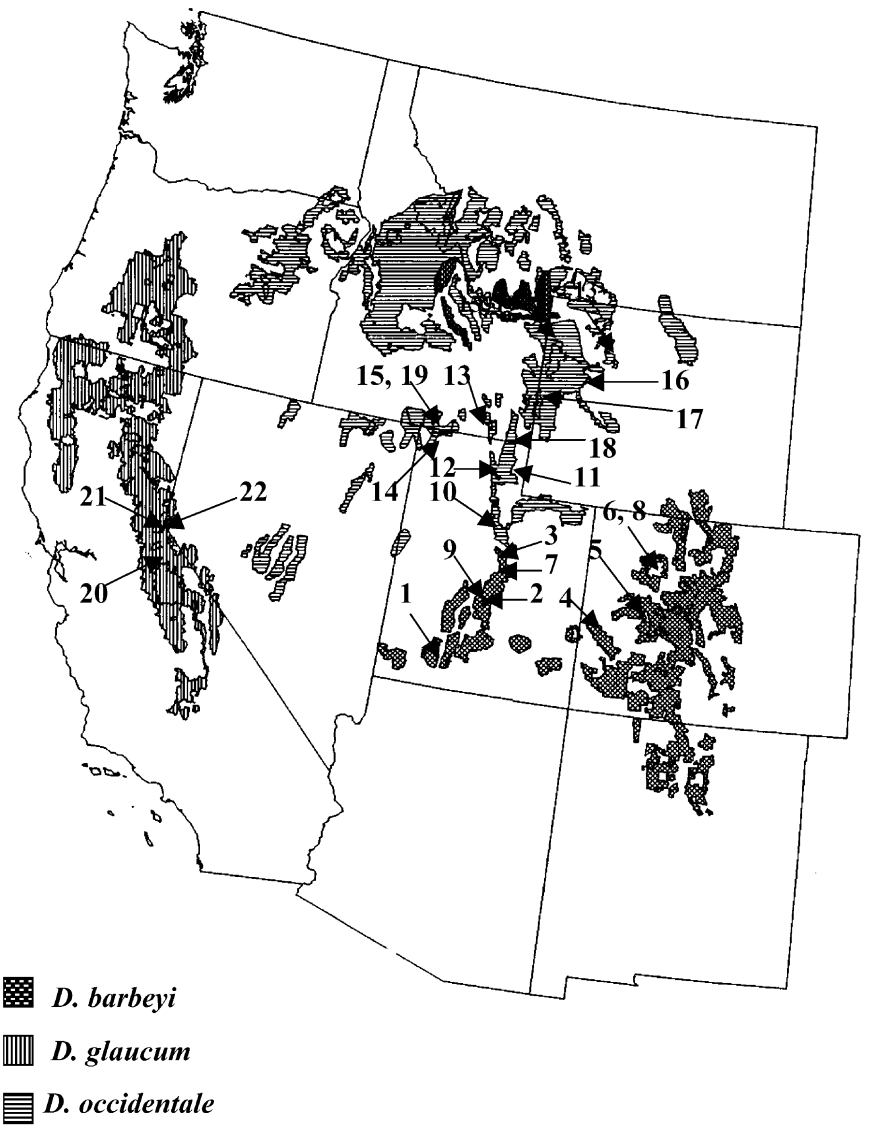

Fig. 1. Geographic distribution of three toxic tall larkspurs in the western US and the location of 22 accessions from *D. occidentale*, *D. glaucum* and *D. barbeyi* collected for molecular genetic study. The extreme northern boundary for *D. glaucum* (also referred to as *D. brownii*) is reported to extend northward into Canada and parts of Alaska (See Welch and Ralphs, part 3, this issue).

2.3. RAPD assay

The decamer oligonucleotides kits were obtained from Operon Technologies. Stoffel fragment of AmpliTaq DNA polymerase, 10× buffer, and 25 mM $MgCl_2$ were

obtained from Perkin–Elmer. Different concentrations of template DNA, primer, $MgCl_2$, Stoffel fragment, and Taq polymerase were tested for optimal amplification products. The optimized amplification reaction mixture (25 μl) contained 13.3 μl sterile ddH_2O, 2.5 μl 10× buffer, 2 μl 8 mM dNTP, 2 μl 10 μM primer, 3 μl 25 mM $MgCl_2$, 0.2 μl (2 units) Stoffel fragment, and 2 μl template DNA (2.5 ng/μl), and was covered by 30 μl of mineral oil. The PCR reaction was performed with the GeneAmp PCR System 9700 for 40 cycles of 93°C for 1 min, 35°C for 1 min, 71°C for 2 min, and stored at 4°C. The amplification products were separated in a 2% agarose gel (Agarose for the separation of GeneAmp PCR products, Perkin–Elmer) containing 0.5 μg ethidium bromide in 1× TBE buffer (pH 8.3). Each gel contains DNA fragment size standards and was photographed under UV light.

2.4. Cloning of RAPD markers for southern hybridization and DNA sequencing

DNA of RAPD markers identified for each species was recovered from low-melting agarose gels and cloned into the PCR 2.1 vector following instructions provided with the Original TA Cloning Kit (Invitrogen). Six to ten white transformants were analyzed for presence of inserts by restriction mapping. Plasmid DNA with correct inserts was labeled with digoxigenin by PCR-labeling methods and subsequently used as probes in Southern blot hybridization to verify their species specificity. The DNA hybridization was detected by anti-digoxigenin-AP, NBT, and BCIP (Boehringer Mannheim). Some species-specific markers were sequenced with an ABI 373A DNA sequencer.

2.5. Data analysis

Reproducible bands were scored for their presence (1) or absence (0) in 184 individuals of 22 accessions. A dendrogram was generated based on the presence and absence of 144 polymorphic RAPD markers using SAHN clustering of NTSYS-PC, version 2.02 (Rohlf, 1992), by the unweighted paired group mean arithmetic average (UPGMA) method. Similarity coefficients were generated and analyzed using Similarity for Qualitative Data (SIMQUAL).

3. Results and discussion

3.1. Screening and identification of species-specific RAPD markers

Bulked DNA from eight individuals of the species *D. occidentale*, *D. glaucum*, and *D. barbeyi* collected from Oakley, ID, Carson Pass, CA, and Manti, UT, respectively, in 1997, were used for screening 140 randomly selected decamer primers. From the results of this preliminary survey, 23 RAPD primers were selected based on the production of reproducible amplified bands for each species. Analysis of the 184 *Delphinium* samples using the selected RAPD primers amplified a total of 188 products, of which 144 were polymorphic. There was an average of 8.2 RAPD pro-

ducts per random primer. Ten bands were shared by *D. glaucum* and *D. occidentale*, eight shared by *D. barbeyi* and *D. glaucum*, and 18 shared by *D. occidentale* and *D. barbeyi* (Table 2). Fifty bands were species-specific (13 for *D. occidentale*, 18 for *D. glaucum*, and 19 for *D. barbeyi*) (Table 2) and the remaining 58 bands were specific for individual accessions.

3.2. RAPD marker variations within and among the three species

Based on the presence or absence of the 144 polymorphic bands, the genetic variations within and among the 22 accessions (184 samples) were measured (see dendrogram, Fig. 2). With the exception of two populations, the Jaccard's genetic similarity coefficients were all >0.9, indicating that little variation existed among individuals within each accession. The similarity coefficients ranged from 0.64 to 0.98 in the hybrid H2 and H3 accessions.

Considerable genetic variation occurred among the different accessions but the three species (identified a priori) were grouped into three distinct clusters. *D. occidentale* and *D. barbeyi* are relatively closer genetically (with a Jaccard's similarity coefficient of 0.64) than either is to *D. glaucum* (with a Jaccard's similarity coefficient of 0.55).

Morphologically, *D. occidentale* is similar to *D. glaucum*, in flower shape and color, lack of pubescence on the stem (except in the inflorescence), and leaf structure; however, they are farthest apart genetically (Fig. 2). *D. barbeyi* and *D. occidentale* appear genetically close, but morphologically distinct. The molecular genetic data and geographic relationships are in agreement with those based on the historical morphological characteristics (Ewan, 1945) and are contrary to the more recent relationships upon which Warnock (1995) proposed to combine *D. occidentale* with *D. glaucum* into one species.

The genetic closeness of *D. barbeyi* and *D. occidentale* was further evidenced by the existence of interspecific hybrids between these two species. At least two populations (H2 and H3) had some individuals clustered with both species (Fig. 2). A detailed analysis of species-specific RAPD markers from H2 and H3 populations found nearly equal numbers of *occidentale*- and *barbeyi*-specific RAPD markers. In contrast, individuals in accessions of *D. barbeyi* and *D. occidentale* contained at most only one RAPD marker that was specific to the other species. The accession labeled H1, collected near Yampa, CO, was initially identified as a putative hybrid of *D. occidentale* and *D. barbeyi*. Detailed analysis of the 10 individual samples from this location identified at the most two RAPD markers specific for *D. barbeyi*. Based on the molecular genetic data, this accession could be identified as *D. occidentale*.

The species-specificity of some RAPD markers was verified by Southern blot hybridization using cloned markers as probes. $OPE17_{580}$ is highly specific to *D. barbeyi* (Fig. 3(a)) while $OPK01_{500}$ is *D. barbeyi* specific to a lesser degree (Fig. 3(b)) as this band was weakly amplified in one *D. occidentale* sample. A cloned RAPD marker, when used as the probe, may hybridize with other PCR products of different sizes in addition to the exact marker band from which it was excised for

Table 2
List of primers, their sequences, and amplification results of RAPD reactions with three tall larkspur species (*o=D. occidentale*, *g=D. glaucum*, and *b=D. barbeyi*) as the template

Primer	Sequence 5′————3′	Total bands	Number of reproducible polymorphic bands (shared and species-specific)					
			o-g	*g–b*	*o–b*	*o*	*g*	*b*
OPA11	CAATCGCCGT	8		A11-670			A11-280	A11-950
OPA16	AGCCAGCGAA	8	A16-930		A16-380			
OPF08	GGGATATCGG	12	F08-550			F08-290		F08-700, 500
OPH03	AGACGTCCAC	9		H03-600	H03-400	H03-700	H03-420	
OPG12	CAGCTCACGA	13	G12-450	G12-400	G12-700	G12-600	G12-650	G12-550
OPG09	CTGACGTCAC	11	G09-580, 490	G09-400	G09-500, 280			
OPH05	AGTCGTCCCC	8			H05-690		H05-720, 610, 320	H05-300
OPI03	CAGAAGCCCA	4				I03-580	I03-720	
OPE17	CTACTGCCGT	5	E17-850					E17-580
OPK01	CATTCGAGCC	9			K01-1000, 350	K01-420		K01-500
OPK08	GAACACTGGG	7	K08-280		K08-470	K08-380	K08-570	K08-800
OPK10	GTGCAACGTG	9		K10-550	K10-1000, 320	K10-490, 390	K10-600, 350, 290	
OPK13	GGTTGTACCC	8		K13-320		K13-350, 280		K13-550
OPO07	CAGCACTGAC	8		O07-620		O07-450	O07-750	O07-900
OPC04	CCGCATCTAC	9	C04-350		C04-500, 450		C04-400	C04-380
OPC05	GATGACCGCC	6	C05-300	C05-900	C05-700			
OPC10	TGTCTGGGTG	4			C10-620			C10-360
OPO10	TCAGAGCGCC	6			O10-600			O10-280
OPH17	CACTCTCCTC	8					H17-350	
OPF13	GGCTGCAGAA	11						F13-650, 600
OPI13	CTGGGGCTGA	8	I13-490		I13-630		I13-700	I13-350
OPG16	AGCGTCCTCC	10			G16-600	G16-370	G16-650	G16-850
OPE04	GTGACATGCC	7				E04-380	E04-600, 420	E04-900, 350
Total		188	10	8	18	13	18	19

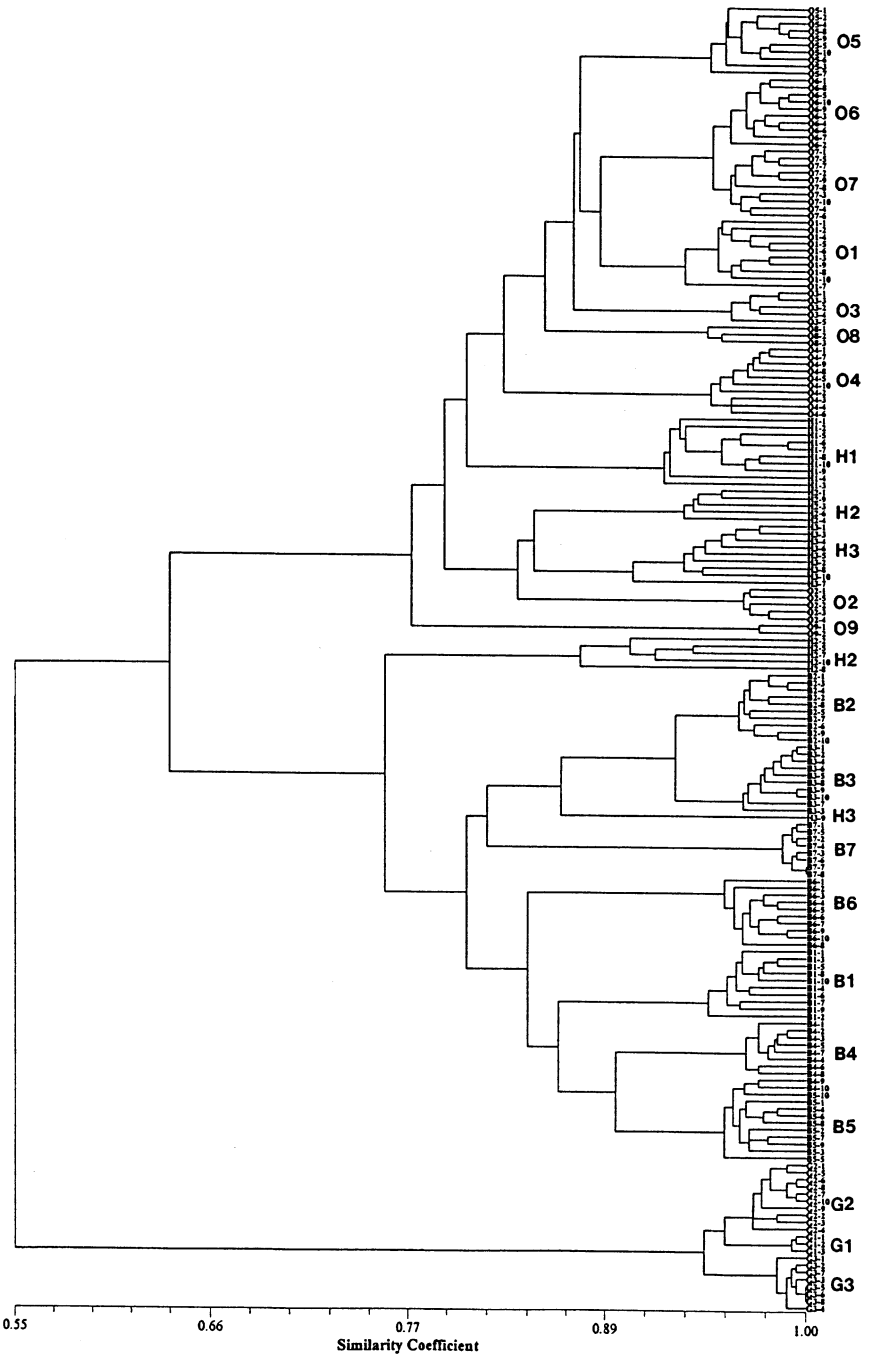

Fig. 2. A dendrogram depicting genetic relationships within and among 22 accessions of *D. occidentale*, *D. glaucum*, and *D. barbeyi* based on 144 RAPD markers.

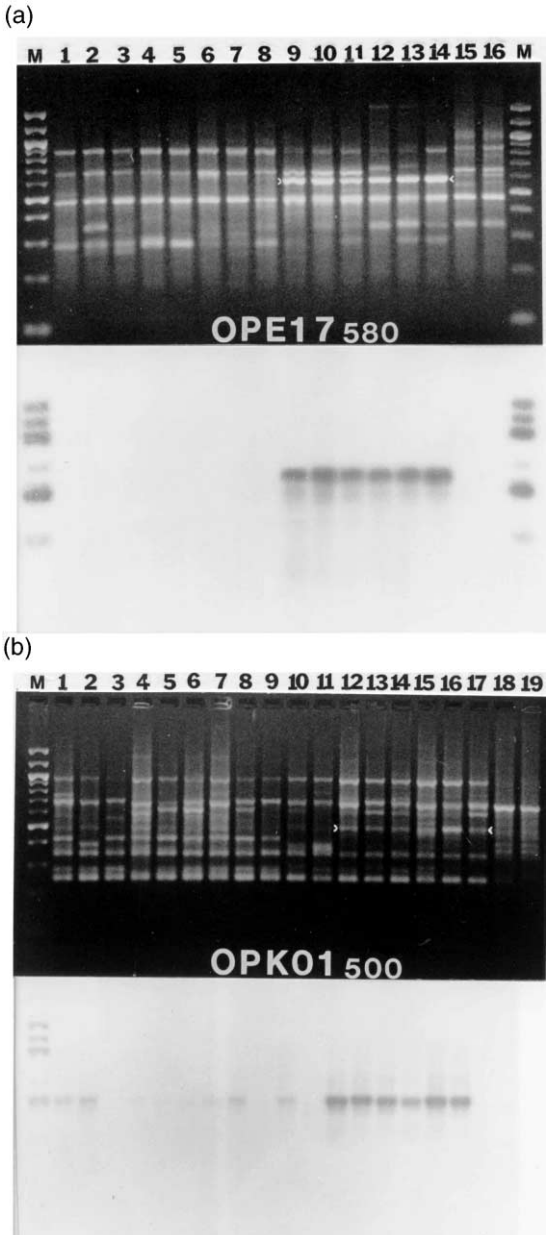

Fig. 3. RAPD profile (top) and Southern blot hybridization (bottom) of RAPD markers specific to *D. barbeyi*. (a) $OPE17_{580}$, lanes 1–8: *D. occidentale*; lanes 9–14: *D. barbeyi*; lanes 15–16: *D. glaucum*. (b) $OPK01_{500}$, lanes 1–8: *D. occidentale*; lanes 9–11: interspecific hybrids of *D. occidentale* and *D. barbeyi*; lanes 12–17: *D. barbeyi*; lanes 18–19: *D. glaucum*.

cloning. Partial homology may also be shared by RAPD products of similar sizes in different species, e.g. weak hybridization with one band in *D. occidentale* probed by $OPK01_{500}$ (Fig. 3(b)). The primer OPK08 produced one RAPD fragment, of 570 bp in length, that was specific to *D. glaucum* (Fig. 4). Specificity to *D. occidentale* was confirmed for $OPH03_{700}$ (Fig. 5(a)) and $OPG12_{600}$ (Fig. 5(b)).

Our study demonstrates that RAPD markers are useful in unambiguous separation of the three larkspur species, even to the extent that accessions within a species could be distinguished by the RAPD technique. Species-specific RAPD markers were identified that will be useful in the identification of particular larkspur species and putative interspecific hybrids in future studies. The distinctiveness between *D. occidentale* and *D. barbeyi* was so great that giving them subspecies status under one species (i.e. *D. occidentale* ssp. *occidentale* and ssp. *barbeyi*, Welsh et al., 1987) is not warranted, nor is the combining of those plants that have been historically identified as *D. occidentale* to be classified as *D. glaucum* (Warnock, 1995). We propose that the original classification of Ewan (1945) separating the three distinct species is genetically correct. Furthermore, the concentration of alkaloids in the plant (Manners et al., 1995) and their toxicity to cattle (Nation et al., 1982; Pfister et al., 1994a,b) merits different management recommendations (Pfister et al., 1997; Ralphs et al., 1997).

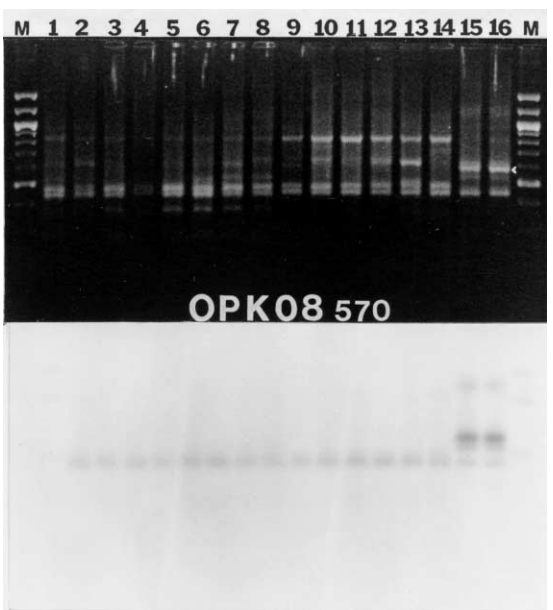

Fig. 4. RAPD profile (top) and Southern blot hybridization (bottom) of the RAPD marker $OPK08_{570}$ specific to *D. glaucum*. Lanes 1–8: *D. occidentale*; lanes 9–14: *D. barbeyi*; lanes 15–16: *D. glaucum*.

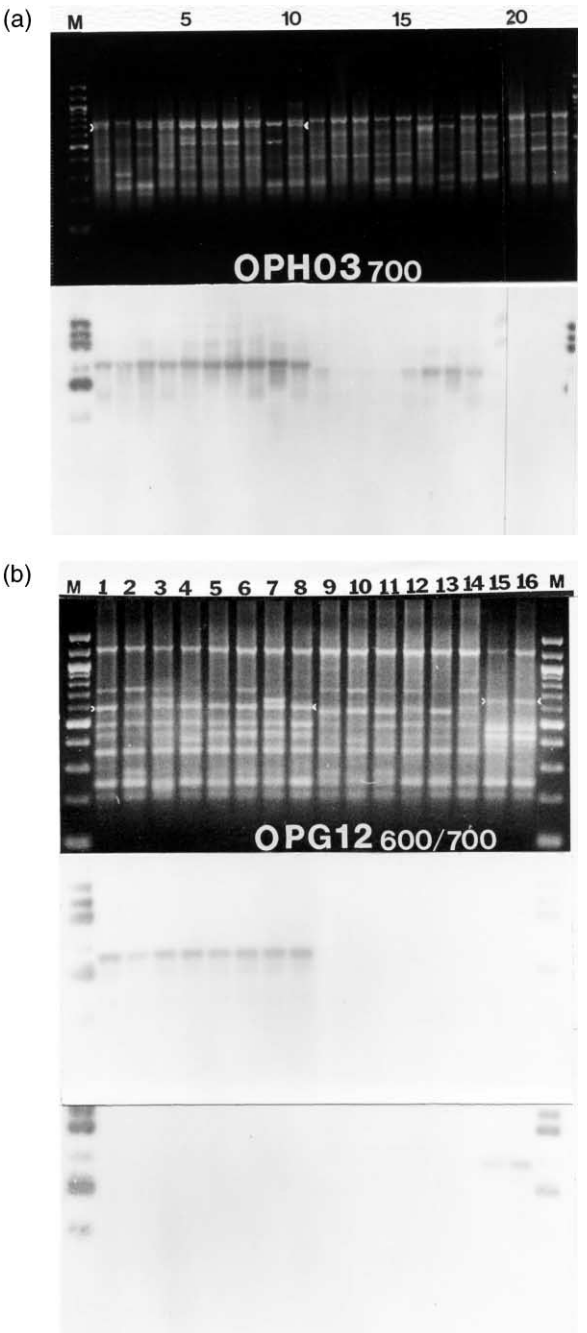

Fig. 5. RAPD profile (top) and Southern blot hybridization (bottom) of RAPD markers specific to *D. occidentale* (a) $OPH03_{700}$, lanes 1–9: *D. occidentale*; lanes 10–12: interspecific hybrids of *D. occidentale* and *D. barbeyi*; lanes 13–19: *D. barbeyi*; lanes 20–22: *D. glaucum*. (b) $OPG12_{600}$ for *D. occidentale* and $OPG12_{700}$ for *D. glaucum*, lanes 1–8: *D. occidentale*; lanes 9–14: *D. barbeyi*; lanes 15–16: *D. glaucum*.

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